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Full Length Research Paper

Phenotypic and plant growth promoting characteristics of *Rhizobium leguminosarum bv. viciae* from lentil growing areas of Ethiopia

Mulissa Jida[1,2]* and Fassil Assefa[1]

[1]Microbial, Cellular and Molecular Biology Program, Faculty of Life Science, Addis Ababa University, Ethiopa.
[2]Biology Program, Faculty of Natural Science, Wollega University, Ethiopia.

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Lentil (*Lens culnaris* Medik.) is an invaluable source of protein for the vast majority of Ethiopian people. It also maintains soil fertility through symbiotic nitrogen-fixation in association with *Rhizobium leguminosarum* bv. v*iciae.* The aim of this study was selecting symbiotically efficient native lentil nodulating rhizobia endowed with different plant growth-promoting (PGP) characteristics. Hence, 30 lentil nodulating rhizobial isolates were isolated from soils collected from different farmer fields found in Central and Northern parts of the country. All isolates were characterized based on morphological, physiological, symbiotic and PGP characteristics. The result of this study showed that these isolates have exhibited interesting features such as wide range of carbon-sources and nitrogen sources utilization, tolerance to acidic and alkaline pH, metal toxicity and antibiotics. Symbiotic characterization indicated that all tested isolates have showed great diversity in their capacity to nodulate their host plant and produce shoot dry matter yield under glasshouse conditions. In addition, they showed PGP characteristics such as IAA production and inorganic phosphate solubilization. Out of all tested isolates 36.7% of them were IAA producer while only 16.7% were insoluble inorganic phosphate solubilizer. In general, from the present study, it can be concluded that Ethiopian soils harbor highly efficient nitrogen-fixing lentil nodulating rhizobia which are diverse in their morphological, physiological and symbiotic characteristics.

Key words: Lentil, *Rhizobium*, Ethiopia, IAA production, phosphate solubilization.

INTRODUCTION

Lentil (*Lens culnaris* Medik.) is an important pulse crop grown widely through out the world (Ford and Taylor, 2003; Erskine, 1997). It is also one of the principal cool season food legumes which have been widely grown in Ethiopia over an area of 90,000ha with annual production of 55,000 tones; the average national productivity being about 0.6 t/ha (CSA, 2004) which is very low compared to its potential (Hailemariam and Tsige, 2006). Nutritionally lentil is an invaluable source of protein (23 to 24%) (Werner, 2005) for the vast majority of Ethiopian people. It is consumed in different preparations, as a split or whole grain with the cereal 'injera '(pan cake) and sometimes as roasted or boiled whole grain snack alone or often mixed with cereals. The relatively high level of lysine in lentil compensates for low concentration in cereal grains, hence when consumed in combination gives nutritionally well balanced diet (Muelbauer et al., 1985) and thus contribute for food security. Thus, improving the production of this crop contribute to the food security of this country. Besides, lentil is leading in fetching the local market price and comparably has significant export market option out of field crops (Fikre and Bejiga, 2006). The straw/haul is also an important source of feed for animals fattening.

In addition to nutritionally quality and source of cash, lentil restores and maintains soil fertility through its symbiotic nitrogen-fixation in association with *R. leguminosarum* bv. v*iciae* (Vincent, 1970; Jordan, 1984;

*Corresponding author. E-mail: mulissa2003@yahoo.com.

Somasegaran and Hoben, 1994). This legume symbiotic nitrogen fixation may not be panacea, but it is often the most feasible and economical N input for resource-poor Ethiopia farmers (Hailemariam and Tsige, 2006). It is capable of supplying 80 kg N ha^{-1} to the soil (Werner, 2005). Consequently, it is grown in rotation with major cereals such as tef (*Erograstic tef*), wheat (*Triticum sp.*), barley (*Horduem vulgare*) and others as restorer of soil fertility instead of using chemical fertilizer for its production. Hence, its yield has remained very low and thus many researches have been undertaken to improve lentil cultivars with respect to their yield, tolerance to different diseases and environmental stresses (Fikiru et al., 2007; Fikre and Bejiga, 2006; Bejiga and Anbessa, 1999, 1995). Nevertheless, these alone could not improve the extremely low productivity of lentil. One of the measures which have been given less attention was exploiting the benefits of its symbiotic nitrogen fixation by manipulating its microsybiont since this can be achieved only if the host is nodulated by effective *Rhizobium* strains (Wolde-maskel, 2007). Therefore, research attention should also be given to characterization, selection and utilization of its rhizobia to improve the amount of nitrogen fixed so as to increase the yield.

Recently, some *R. leguminosarum bv.viciae* strains are considered as plant growth promoting rhizobacteria (PGPR), since they can promote the growth of non-legumes through mechanisms that are independent of biological N_2 fixation (Antoun et al., 1998; Yanni et al., 2001; Sommers and Vanderleyden, 2004). Rhizosphere bacteria that are capable of stimulating plant growth by colonizing roots are known as PGPR (Kloepper, 1993). PGPR stimulate plant growth directly either by synthesizing phytohormones such as indole-3-acetic acid (IAA) or by promoting nutrition processes such as phosphate solubilization and siderophore production, which facilitate phosphorus and iron uptake, respectively from soil (Kloepper, 1993; Lippmann et al., 1995). They can also stimulate growth indirectly by protecting the plant against soil-borne fungal pathogens (O'Sullivan and O'Gara, 1992). Several studies reported that *Rhizobium leguminosaru* bv.*viciae* share characteristics such as phosphate solubilzation, phytohormones and siderophore production, and biocontrol activity with PGPR strains (Antoun et al., 1998; Yanni et al., 2001; Vergas et al., 2009). Such kind of rhizobial strains could be used as broad-range (Wolde-maskel, 2007) multipurpose inoculants for both legume and non-legume crops grown rotationally or subsequently. Thus, local rhizobial isolates must be screened for their plant growth-promoting (PGP) activity in addition to their excellent symbiotic performance (Wolde-maskel, 2007).

R. leguminosarum bv. v*iciae s*trains, the microsymbiont of lentil symbiosis naturally vary in their nitrogen fixing capacity and adaptation to prevailing environmental stresses (Zharan, 1999). Consequently, selection of symbiotically efficient rhizobial strains which are tolerant to locally prevailing stresses has paramount importance.

In other parts of the world where lentil is commonly grown several studies were conducted on lentil rhizobia characterization and selection of best strains for inoculant production (Harun et al., 2009; Sajjad et al., 2008; Athar, 1998). Athar (1998) studied drought pattern of lentil rhizobia isolated from arid and semiarid areas and indicated that strains originated from saline areas showed significantly better survival under low water potential. In addition, Sajjad et al. (2008) studied the genetic diversity of lentil nodulating rhizobia isolated from Punjab, Pakistan by using random amplified polymorphic DNA (RAPD) markers and obtained a considerable genetic diversity among them. Harun et al. (2009) also characterized lentil rhizobia using both physiological and molecular methods and showed that there was a considerable variation among different strains.

However, very little attention has been given to lentil rhizobia characterization and utilization in Ethiopia. These necessitates the need for research activities devoted to isolation and characterization of lentil root nodule bacteria which eventually leads to the selection of strains which are adapted to local environmental conditions. Hence, this study was initiated with the aim of developing well characterized culture collection of lentil nodulating rhizobia which are symbiotically efficient and endowed with different PGP attributes.

MATERIALS AND METHODS

Study sites and soil samples collection

Soil samples were collected from the lentil grown farmer's field found in Showa, Gonder, Gojam, Wallo and Tirgay areas of Ethiopia, which are the major lentil producing areas of the country (Table 1). The areas are distributed in central and northern parts of the country (Figure 1). The areas are distributed on altitudes between 1800 to 3100 m above sea level (m a.s.l.), with soil pH ranging from moderately acidic to neutral or slightly alkaline.

Thirty-five samples were collected in October, 2009. Lentil agricultural fields which have lentils with good growth and healthy appearance and with no previous history of inoculation with rhizobia were selected from each site and soils were excavated from the upper 15 to 20 cm depth, pooled together and composite samples were collected in sterile plastic bags. A bout three kg of composite soil samples collected from each sites were carefully transported to the Applied Microbiology laboratory, Addis Ababa University, for further work.

Isolation of rhizobia

Rhizobia were isolated from soil samples by inducing nodulation on lentil cultivar called 'Gudo' obtained from Debera Zeit, Ethiopian Agriculture Research Organization (EARO) using plant infection method (Vincent, 1970). Each soil sample was thoroughly mixed and air-dried in a glasshouse. Air-dried soil samples were ground and passed through a 2 mm sieve to remove stones and large pieces of organic matter, and then filled into 3 kg capacity surface sterilized (96% ethanol) plastic pots.

Lentil seeds were selected and surface sterilized with 95% ethanol and 0.1% acidified mercuric chloride solutions for three minutes with each (Vincent, 1970) and rinsed five times with

Table 1. Lentil rhizobila isolates, site of isolation, growth and colony characteristics.

Isolate	Name of isolation site	Altitude of isolation site (masl)	pH of isolation soil	Colony characteristics	Colony diameter (mm)	MGT(h)	YEMA BTB color
LR1	ShenoII	2868	5.58	LMLM	2.6	3.7	Y
LR2	Ambo	2170	6.75	LCM	3.1	2.5	Y
LR3	Keyit	2931	6.38	LMM	3.9	2.1	Y
LR4	Wajel	2432	6.12	LCLM	3.1	2.6	Y
LR5	Sandafa	2554	6.0	LMLM	3.3	2.7	Y
LR6	D/Selam	1896	6.0	LCM	3.7	1.9	Y
LR7	Fogera meda	1799	6.0	LCM	3.6	2	Y
LR8	Tefki	2047	6.55	LCM	3.1	2.8	Y
LR9	Makesanyit	1978	6.39	LWM	2.5	3.4	Y
LR10	Yetinora	2437	6.21	LWM	3.0	2.3	Y
LR11	Teji	2065	6.7	LCM	3.2	2.2	Y
LR12	ShenoI	2032	5.58	LCM	3.3	2.1	Y
LR13	Asgori	2078	6.2	LCM	2.8	2.9	Y
LR14	Chatawa	2912	5.12	LWM	2.9	2.3	Y
LR15	CholeII	2647	6.05	LCM	3.1	2.4	Y
LR16	Tikana	1942	7.18	LWM	3.2	2.5	Y
LR17	Wuchale	1980	6.58	LMM	3.1	2.6	Y
LR18	Obbi	2108	6.61	LWLM	2.5	3.8	Y
LR19	Gurura	1906	7.01	LCM	3.3	2.3	Y
LR20	CholeI	2612	5.58	LWM	3.4	2.4	Y
LR21	Asketema	2499	7.04	LCM	3.6	2.2	Y
LR22	Dagam	3101	6.36	LWM	2.6	3.5	Y
LR23	Goha Tsion	2517	6.3	LCM	3.5	2.1	Y
LR24	Woliso	2008	5.58	LWM	3.4	2	Y
LR25	Adanaba	2533	7.03	LMLM	2.6	3.6	Y
LR26	Fiche	2748	6.83	LWM	3.1	2.3	Y
LR27	Korem	2482	6.93	LWM	3.0	3.2	Y
LR28	Geshana	2907	5.8	LWM	3.2	2.4	Y
LR29	Ginchi	2250	6.60	LWM	3.0	2.4	Y
LR30	Fala'a	2549	7.0	LWM	3.1	2.8	Y

LCM: large, creamy, mucoid; LWM: large, watery, mucoid; LMLM: large, milky, less mucoid; LWLM: large watery, less mucoid; LCLM: large, creamy less mucoid; Y: yellow.

sterilized distilled water to remove traces of sterilizing chemicals. Surface sterilized seeds were allowed to germinate on sterile water gar (1%) surface for three days at 25°C and five pre-germinated seeds were planted on each pot, which were thinned down to three after 5 days of emergence (DAE). All pots were situated in glasshouse over the table and watered to a field capacity every three days for 45 days after planting (DAP).

Forty-five DAP all the plants were carefully uprooted from the pots and washed under gently flowing tap water to remove soil particles. Large and pink nodules were separately collected from each pot on separate sterile Petri dishes and surface sterilized as described before, and crushed using alcohol flamed glass rod. Loopful of the extract was streaked on Yeast extract Mannitol Agar (YEMA) containing 0.0025% (w/v) Congo red (CR) (Vincent, 1970). The components of YEMA g/L: 0.5 K_2HPO_4, 0.2 $MgSO_4$, 0.1 NaCl, 10 Mannitol, 0.5 Yeast Extract, 15 Agar (Vincent, 1970). All the plates were incubated at 28±2°C for 3-5 days. From each plate, single typical rhizobia colony were picked and transferred to test tubes which contain sterile Yeast extract Mannitol Broth (YEMB) (Vincent, 1970). The test tubes were incubated at 28 ±2°C for 3 days and purified by re-streaking on new YEMA plates for growth. The pure cultures were further confirmed by presumptive tests such as gram reaction and growth on Peptone glucose agar (PGA) (Vincent, 1970). Pure isolates were then preserved on YEMA slants containing 0.3% $CaCO_3$ stored at 4°C for short-term storage (Vincent, 1970) and 50% glycerol at -20°C for long-term storage. All the rhizobial isolates were designated as LR1-30(L: Lentil; R: Rhizobia) (Table 1).

Authentication and preliminary symbiotic characterization of the Isolates

All rhizobial isolates were authenticated by re-inoculating on their hosts. Simultaneously their symbiotic effectiveness of the strains was also evaluated in sand culture. About 3 kg of acid washed and autoclave sterilized river sand was placed in plastic pots (3 kg capacity). The lentil cultivar called 'Gudo' was used in this study. Lentil seeds were selected and surface sterilized and germinated

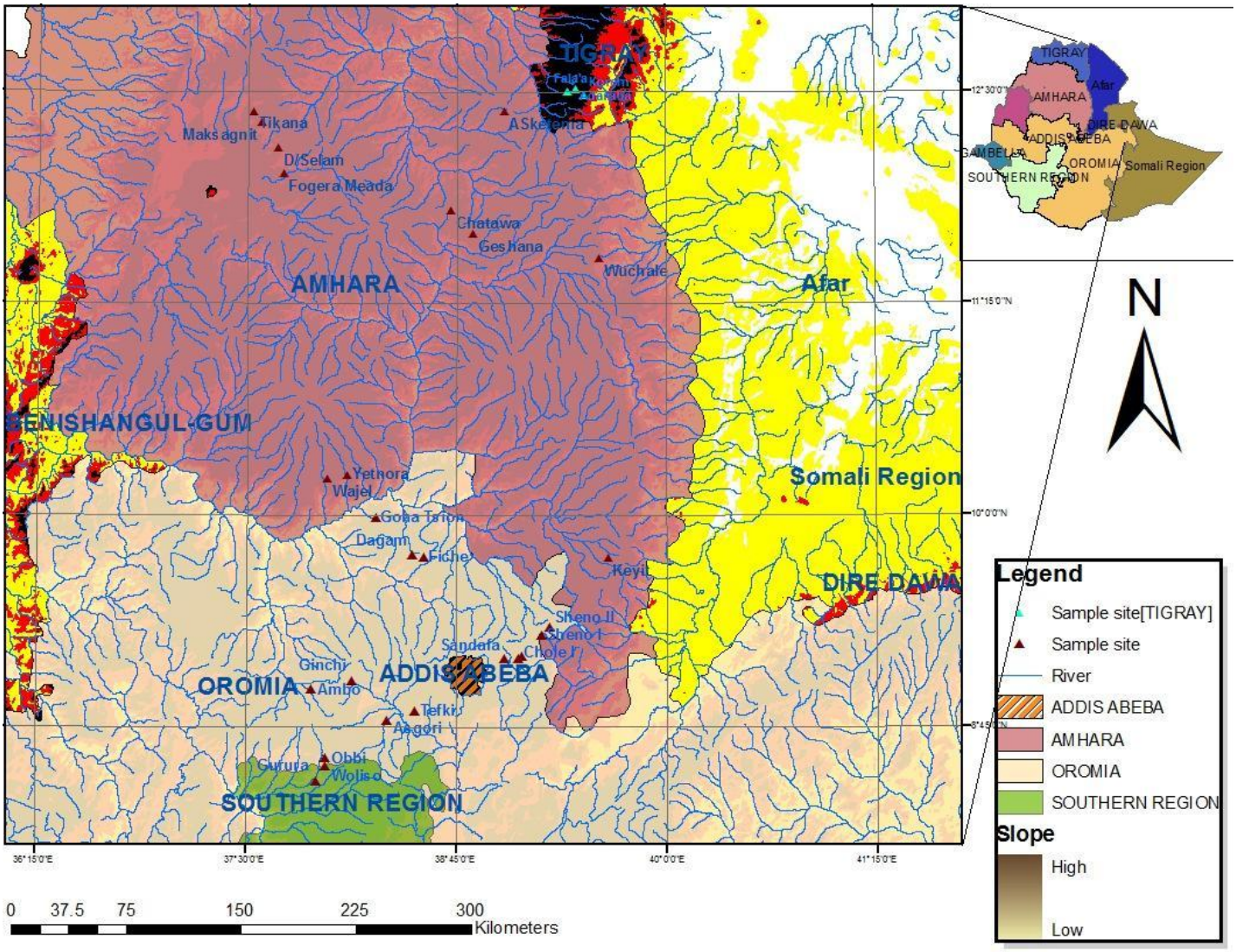

Figure 1. Soil sample collection sites.

as described before. All rhizobial isolates were cultured in YEMB tubes for three days. Germinated seedlings on the agar surface were flooded with each rhizobial culture adjusted 10^9 cells per seed for one hour on separate plates. Five inoculated seedlings were transferred on each pot which was latter on thinned down to three after 5 DAE. The pots were irrigated with nitrogen free plant nutrient solution as described by (Somasegaran and Hoben, 1994). Uninoculated nitrogen-fertilized pots were included as positive (TN) control and uninoculated (T0) pots as negative control. The experiment was statistically laid out with three replications using randomized block design. As control, each block contained two pots (T0 and TN) with uninoculated seedlings. Plants were supplied with distilled water every three days, and they were saturated once a week with a nitrogen-free nutrient solution (Somasegaran and Hoben, 1994). Furthermore, TN control received weekly 0.05% (w/v) KNO_3 as nitrogen source weekly while all other pots received this solution initially as starter nitrogen only once. The experiment was set in triplicate. Sixty DAP all seedlings were carefully uprooted, and nodule number counted, and nodule dry weight and shoot dry weight were determined after drying at 70°C for 48 h to the constant weight.

Colony morphology, growth and biochemical characterizations

Colony morphology (colour, mucoidty, transparency, and borders) was evaluated by streaking a loop of the initial inoculum on YEMA plates and allowing the bacteria to grow at 28°C for 5 days (Vincent, 1970; Somasegaran and Hoben, 1994). Bacterial growth was assessed on YEMB incubated in a gyratory shaker at 120 rev.min^{-1}, by measuring the optical density at 540 nm every 6 h and spread plating 0.1 ml diluted culture on YEMA. The generation time (GT) was calculated from the logarithmic phase of growth. Acid or alkali production test which was carried out by growing isolates on YEMA medium containing bromothymol blue (BTB) (Somasegaran and Hoben, 1994).

Physiological characteristics

All tests, except carbohydrate and N-source assimilation, were carried out on YEMA plates. Petri dishes containing YEMA medium were subdivided into squares and each square was inoculated with 10 µl of 72 h bacterial YEM broth. After 5 days of incubation at

28°C, bacterial growth was compared to the controls. Three replicates were included per treatment.

Salt, pH and temperature tolerance

Salt tolerance of the isolates was determined on YEMA plates containing from 0 to 2% (w/v) NaCl concentrations. Tolerance to extreme pH was tested on YEMA medium set at different pH values using 1 NHCl and 1 N NaOH. Temperature tolerance was evaluated on YEMA plates inoculated as described above and incubated at temperatures from 4 to 40°C.

Intrinsic antibiotic and heavy metal resistance

This intrinsic antibiotic and heavy metal resistance was determined on solid YEM medium containing the following filter sterilized antibiotics or heavy metals (µg.ml –1): ampicillin (5 and 10), chloramphenicol (5 and 10), erythromycin (5 and 10), nalidixic acid (5 and 10), rifampcin (5 and 10), streptomycin (10 and 50), neomycin(5 and 10) and tetracycline (5 and10); $AlK(SO_4)_{3.}12H_2O$ (10, 25), $K_2Cr_2O_7$ (50), $CoCl_2$ (10), $CuCl_2,2H_2O$ (10), $HgCl_2$ (5), $MnCl_2$ (50, 75), $NiSO_4$ (10), $Pb(CH_3COO)_2$ (10), and $ZnCl_2$ (50).

Utilization of different C-sources and N-sources

Inocula of the tested isolates were obtained from 72 h YEMB test tubes and the plates were inoculated as described above. Different carbohydrates were added as described by Amarger et al. (1997) at final concentration of $1gmL^{-1}$ to the basal medium containing (gmL^{-1}): K_2HPO_4, 1; KH_2PO_4, 1; $FeCl_3$. 6H2O, 0.01; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.1; NH_4SO_4, 1; and 15 gm of agar. The following filter sterilized (0.22µm milipore) as sole carbon sources were added after autoclaving: Citrate, D-Sorbitol, D-mannose, D-maltose, D-galactose, D-fructose, α-lactose, D-arabinose, gluconate, D-glucose, raffinose, xylose, dulcitol, cellobiose, glycerol, anoditol, inulin, aesculin, D-mannitol, trehalose, inositol, α-cellulose, sucrose and dextrin were added after autoclaving. Filter sterilized L-Tryptophan, Methionine, L-Tyrosine, Leucine, L-Asparagine, DL-β-Phenylalanine, L-Arginine, Glutamic acid, L-Lysine, Analanine, Serine, Glycine, Thiamine, Niacin and Riboflavin were used as sole nitrogen source for isolates by adding a final concentration of 0.5 gm/L to the above basal medium from which NH_4SO_4 was omitted and mannitol was added (Amarger et al., 1997). Inoculated palates were incubated at 28°C and results were observed after 3-5 days.

Determination of PGP properties of the Isolates

Phosphate solubilization

Phosphate solubilizing (PS) ability of the isolates was determined using Pikovaskya (Pikovskya, 1948) agar plates spot inoculated with a loopful of culture of bacterial isolates adjusted to 10^4 cells ml^{-1}. After incubation at 28±1°C for 5 to 7 days, formation of a clear zone around the spot was recorded and Solubilization index (S.I.) was calculated as the ratio of halo zone diameter to colony diameter for positive isolates.

Screening for in vitro antagonistic activity against fungal pathogens

The *in vitro* inhibition of mycelial growth of *Fusarium oxysporum f.sp. lentis* (obtained from Debere Zeit, EARO) by the rhizobial isolates was tested using the dual culture technique as described by Paulitz et al.(1992) and Landa et al.(1997). Equal number (10 µl) of each isolates was equidistantly placed on the margins of YEMA plates amended with sucrose (0.5%) and incubated at 28°C for 24 h. A 4-5 mm agar disc from PDA cultures of the fungal pathogens will be placed at the centre of the YEMA plate for each bacterial isolate and incubated at 28 ± 2°C for 5-7 days.

IAA production

To determine indole- 3-acetic acid (IAA) production, exponentially grown cultures (10^8 cells ml^{-1}) of the strains were incubated separately on broth medium amended with 5 mM L-tryptophan for 24 h (Bric et al., 1991). Supernatant of the strains were collected by centrifugation at 10,000 rpm for 15 min and 2 ml supernatant of each was transferred separately to a fresh tube to which 100 µl of 10 mM O-phosphoric acid and 4 ml of Salkowaski reagent (1 ml of 0.5 mM $FeCl_3$ in 35% HClO4) were added. Mixtures were incubated at room temperature for 25 min and observed for pink color production.

Data analysis

All experiments were set in triplicate and the data is average of three. Symbiotic data was analyzed by analysis of variance and the treatment means will be compared following Duncan's test (DT) by using using SPSS (V.17). Correlations among some parameters were checked by using bivariate regression analysis using SPSS.

RESULTS AND DISCUSSION

Symbiotic characteristics

All tested isolates of lentil rhizobia showed great diversity in their capacity to induce nodule formation on the host plant and produce nodule dry weight under glass house conditions. The mean nodule number per plant was ranged from 20 for isolate LR15 and LR29 to 62 for isolate LR26 (Table 2). Generally, 83% of the isolates formed more than 30 mean nodule number on the roots of their host. Harun et al. (2009) also reported that all tested lentil rhizobia nodulated their host very well with different level of infectivity. The highest nodule dry weight was recorded for isolate LR4 i.e. 112 mg whereas the smallest was 9 mg which was recorded for isolate LR28 (Table 2).

Similarly, all tested isolates of lentil rhizobia exhibited high diversity in their shoot dry matter accumulation. In comparison with TN control which represents 100% level of shoot dry matter and T0 control which represents 34%, all tested isolates showed shoot dry matter yield ranging from 0.32 to 0.73 gm per plant which is higher than T0 control (0.25 gm per plant). The relative effectiveness, which expressed in percent of TN control, showed that isolates LR2, LR8 and LR25 were the most efficient with 100% dry matter yield while LR9, LR12, LR14 and LR17 were the least efficient with less than 50% relative effectiveness (Table 2).The mean comparison showed several overlapping groups for both shoot and nodule dry matter yield and nodule numbers (Table 2). Out of 30 isolates tested 9(33.3%) isolates did not vary significantly

Table 2. PGP activity under *in vitro* and Symbiotic characteristics under glasshouse conditions of the rhizobial isolates.

Treatments	Nodule Number ± SE	Nodule dry weight(mg) ± SE	Shoot dry weight(g) ± SE	Relative effectiveness %	P.S. activity(S.I)	IAA production
LR1	49.5±4.3a-d	74±6a-g	0.71±0.02a-c	97.3	-	-
LR2	39.8±3.6b-g	33±3c-g	0.73±0.1a	100	-	+
LR3	34.7±2.6c-h	50±3a-g	0.41±0.07h-k	56.2	-	-
LR4	34.5±4.4c-h	112±99a	0.66±0.03a-d	90.4	-	-
LR5	57.7±4.6a-c	82±3a-d	0.40±0.02i-k	54.8	-	+
LR6	32.7±2.8d-h	15±3e-g	0.66±0.01a-d	90.4	-	-
LR7	34.7±2.2c-h	25±1d-g	0.45±0.01g-k	61.6	-	-
LR8	32.5±2.6d-h	96±43a-c	0.73±0.05a	100	-	-
LR9	33.0±1.8d-h	11±2e-g	0.35±0.03jk	47.9	-	-
LR10	34.2±0.9c-h	9±3fg	0.57±0.03a-g	78.1	-	-
LR11	38.3±2.2b-h	9±1fg	0.44±0.04g-k	60.3	1.13	+
LR12	27.7±4.2e-h	4±1g	0.32±0.01k	43.8	1.18	-
LR13	40.5±2.7b-f	7±1g	0.59±0.01a-g	80.8	-	-
LR14	34.8±1.9c-h	5±1g	0.34±0.02k	46.6	-	-
LR15	20.3±3.2h	10±1e-g	0.45±0.01g-k	61.6	-	-
LR16	27.2±3.4e-h	10±1e-g	0.44±0.02g-k	60.3	1.14	-
LR17	24.2±2.9f-h	25±8d-g	0.36±0.02jk	49.3	-	+
LR18	52.7±4.9a-c	84±3a-e	0.44±0.01g-j	60.3	-	+
LR19	49.0±7.2a-d	90±12a-c	0.51±0.01df-j	69.9	-	+
LR20	25.7±2.9f-h	17±3e-g	0.46±0.04g-k	63.0	-	-
LR21	47.5±1.3a-d	38±4b-g	0.45±0.04g-k	61.6	-	+
LR22	50.2±6.8a-d	18±1e-g	0.51±0.01d-j	69.9	-	+
LR23	39.3±2.5b-h	15±2e-g	0.56±0.01b-i	76.7	1.15	+
LR24	37.5±2.8c-h	21±3d-g	0.63±0.04a-f	86.3	1.17	+
LR25	45.3±2.9a-e	22±1d-g	0.73±0.01ab	100	-	-
LR26	62.3±4.5a	110±25a	0.67±0.01a-d	91.8	1.13	-
LR27	32.7±3.2d-h	22±3d-g	0.47±0.04f-k	64.4	-	+
LR28	34.3±2.2c-h	9±1fg	0.63±0.03a-f	86.3	-	-
LR29	20.5±0.8gh	15±3efg	0.54±0.02c-i	74	-	-
LR30	33.3±c-h	17±1efg	0.58±0.01a-h	79.5	-	-
TN	-	-	0.73±0.08a	100		
T0	-	-	0.25±0.01l	34.2		

Numbers in the same column followed by the same letter do not differ significantly at p= 0.05 by DT; +: present; -: absent.

from TN control in shoot dry matter yield. Their relative effectiveness ranges from 78 to 100%. These isolates were also particularly most infective with mean nodule number ranging from 32 to 62 and highly effective. These isolates must be taken into consideration for lentil inoculation trail at different zones under Ethiopian soil and climatic conditions. Similarly, Harun et al. (2009) also observed that rhizobial inoculations to the lentil significantly increased shoot dry weight over the uninoculated control.

In our study a correlation between the increase of dry matter and the number or the dry weight of nodules was not found to be statistically significant ($p>0.05$). Similarly, Maâtallah et al. (2002) has also reported that there was no positive correlation between the increase of shoot dry matter and the number and the dry weight of nodules. It has been demonstrated that the dry matter yield was rather correlated with the nodule leghaemogblobin concentration than with the number or the dry weight of nodules (Dudeja et al., 1981).

Growth characteristics and colony morphology

On the basis of their generation times 80% of the isolates were fast growers with mean generation time (MGT)1-3 h, whereas 32% of them were relatively slow growers with 3 h<MGT<4 h (Table 1). Distribution of such rhizobia in acid and neutral to alkaline areas was not correlated with the climatic region, i.e. fast, slow and very slow-growing rhizobia could be found in the same soil (Maâtallah et al., 2002). All isolates formed colony with

Table 3. Physiological characteristics of lentil rhizobial isolates.

Isolate	pH tolerated	NaCl % tolerated	Temperature tolerated	C-sources% utilized	N-sources% utilized	IAR pattern	Heavy metals resistance
LR1	4.5-8.0	1	15-35	92.3%	86.7	Strp,Nal, chol	Mn, Cr
LR2	5.5-8.0	0.75	20-30	88.5	80.0	Nal, Chol	Cr
LR3	5.5-8.0	0.75	20-30	38.5	60	chol	-
LR4	5.5-8.0	0.75	15-35	92.3	86.7	Nal, chol	Cr,Zn
LR5	5.5-8.0	0.75	20-30	30.8	33.3	-	-
LR6	5.5-8.0	0.75	15-35	92.3	78.6	Strp,Nal, chol	Cr
LR7	5.5-8.0	0.75	20-35	92.3	86.7	Chol,Nal	Cr
LR8	5.5-8.0	0.75	20-30	92.3	78.6	Nal, chol	Cr
LR9	5.5-8.0	0.75	20-30	50	60	Amp, chol, Nal,Eryt	Cr
LR10	4.5-8.0	1	10-35	92.3	73.3	Chol, Nal	Mn, Cr
LR11	5.0-8.0	1	20-30	76.9	73.3	Chol	Cr, Zn
LR12	5.0-8.0	1	20-30	46.2	53.3	Chol, Eryt	-
LR13	5.0-8.0	1	20-30	53.8	66.7	-	Cr
LR14	5.5-8.0	0.75	20-30	53.8	46.7	-	-
LR15	4.5-8.0	1	15-35	92.3	66.7	Amp,Chol, Nal, Eryt	Mn, Cr
LR16	5.5-8.5	0.75	20-30	50.0	13.3	Nal, Eryt	-
LR17	4.5-8.0	1	15-35	88.5	86.7	Nal, Eryt	Mn, Cr
LR18	4.5-8.0	1	15-35	88.5	86.7	Amp,Chol, Nal, Eryt	Mn, Cr, Zn
LR19	5.5-8.5	0.75	10-30	88.5	86.7	Nal, Eryt	Cr, Zn
LR20	4.5-8.0	1	15-35	88.5	86.7	-	Mn
LR21	4.5-8.5	1	10-35	92.3	86.7	Eryt	Cr
LR22	5.5-8.0	0.75	20-30	80.8	73.3	Nal, Eryt	Cr
LR23	5.5-8.0	0.75	20-30	61.5	53.3	Nal	-
LR24	5.5-8.0	0.75	20-30	80.8	73.3	Nal	-
LR25	5.5-8.5	0.75	20-30	92.3	86.7	Chol	Cr
LR26	5.5-8.0	0.75	20-30	88.5	86.7	Nal,chol	Cr,Zn
LR27	4.5-8.5	1	15-35	76.9	86.7	Nal	Mn
LR28	5.5-8.0	0.75	20-30	50	46.7	-	-
LR29	5.5-8.0	0.75	20-30	53.8	53.3	-	-
LR30	5.5-8.5	1	20-30	61.5	60	-	-

Amp: ampicillin; Nal; naldixic acid; Eryt; erytheromycin; Chol: chloramphenicol; Strp: streptomycin; -: do not tolerate.

circular shape, entire margin, milky-to-watery translucent to creamy opaque features with different level of mucus production. Most isolates i.e. 86.7% exhibited copious production of exoplosaccharide while the remaining isolates showed less mucus production (Table 1). The colony diameter of the isolates was ranged from 2.5 to 3.9 mm indicating that all isolates could be able to form large colony on YEMA surfaces at optimum pH of growth media (6.7) and incubation temperature 28°C (Table 1). According to Jordan (1984) such characteristics are common among fast growing rhizobia in general and *R. leguminosarum* bv. *viciae* cross inoculation group in particular.

All isolates changed the color of YEMA supplemented with BTB to yellow indicating they are acid producers and hence possible to categorize them as fast-growers. The CR absorption test also indicated that none of the isolates absorbed CR in YEMA plates this is distinctive character of rhizobia with only few exceptions (Somasegaran and Hoben, 1994). On the other hand none of the tested isolates manage to grow on PGA plates. In addition, all tested isolates were KOH test positive indicating that they were gram negative. These indicate that all isolates were not contaminant rather fast growing rhizobia (Vincent, 1970; Somasegaran and Hoben, 1994).

Physiological characteristics

Tolerance of acidic and alkaline pH

As shown in Table 3, the lentil nodulating rhizobia tested showed a wide diversity in their different pH tolerance. All tested isolates grew in mildly acid pH (5.5) to neutral pH and slightly alkaline pH (8.0). At low pH, 26.7% of the isolates exhibited an acid tolerant character since they

grew without restriction at pH 4.5 where as only 13.3% of the isolates grew at pH 8.5 and none of the isolates could grew at pH 9.0. This might be related to less saline nature of the soil from which most isolates were recovered. Fast-growing rhizobia strains which could tolerate 2% NaCl were capable of growing at pH 9.5 (Sadowsky et al., 1983). Similarly, an apparent positive correlation was observed between salt tolerance of the isolates and their alkaline pH tolerance(R=0.78).

Harun et al. (2009) also found that there is great variation among lentil nodulating rhizobia with respect to growth and survival in acidic and alkaline conditions. They showed that they can grow well at acidic pH as low as pH 4 and alkaline pH as high as pH 10. Generally, large variation was observed among fast-growing rhizobial isolates with regard to growth in relation to pH of the medium (Graham and Parker, 1964). There might be a relation between pH of origin of isolates and their acid and alkaline pH tolerance. However, in this study such kind correlation was not statistically significant. Moreover, metal (Al and Mn) toxicity tolerance of isolates was tested at pH5.0 and all tested isolates were found to be sensitive to very low concentration of Al (Table 3). About 23% of them tolerated both high and low concentrations of Mn at pH 5.0. Since most Ethiopian soils are acidic like any other tropical soil where associated metal toxicity expected to prevail such isolates are very important to use as inoculants.

Temperature tolerance

Temperature conditions have a great effect on rhizobial growth and symbiotic performance (Zahran, 1999). As shown in Table 3, maximum growth of all tested strains was obtained between 20 and 30 °C. Below and above those values, the percentage of isolates that grew decreased to reach 10% at 10°C and 46.7% at 35°C. None of the tested isolates could be able to tolerate and grow at 4 and 40°C. Likewise, Harun et al. (2009) observed that all lentil nodulating rhizobia strains from Bangladeshi grew well at 33°C and one strain could grow at 38°C. Increased temperature optima of these isolates may be beneficial for its application in temperature stressed conditions as symbiotic performance of different rhizobial strains under temperature stress has been correlated with their ability to grow in pure culture at elevated temperature (Hungaria, 2000). Though correlation between climatic region of origin area of each isolates and tolerance to low or high temperature was not carried out their temperature tolerance might be related to their origin soil temperature as tropical soil could have wide range of temperature.

Salt tolerance

The data in Table 3 show that lentil rhizobia exhibited less diversity in their salt tolerance. The salt inhibitory concentrations varied among strains. Generally, less tolerance to sodium chloride (NaCl) was observed since 80% of the tested rhizobia could not grow with 1% NaCl. However, at higher concentrations (2%) none of the tested isolates grew. Several studies also reported that rhizobial isolates exhibit a large range of sensitiveness to salinity (Singleton et al., 1982). It is often believed that saline soils naturally select strains more tolerant to salinity. To verify this hypothesis, the growth of lentil rhizobia with various salt concentrations was correlated with their site of isolation soil pH and statistically significant positive correlation(R=0.78) was obtained.

Intrinsic antibiotics and heavy metals resistance

The evaluation of intrinsic resistance to antibiotics of lentil rhizobia showed that most (50 to 80%) of the tested isolates exhibited high resistance to chloramphenicol and nalidixic acid (Table 3). In the presence of ampicillin, streptomycin, or erythromycin, only 3 to 10% (according to antibiotic and their concentrations) of isolates were resistant. Out of all tested isolates and different antibiotics 23.3% of the isolates were found to be sensitive to all tested antibiotics even at low concentration while 56.7% of them resist two and above types of antibiotics. All tested isolates were found to be sensitive to low concentration of tetracycline, neomycin and rifampcin. Previous study (Harun et al., 2009) also observed great variation among lentil rhizobia with respect to their in intrinsic antibiotics resistance pattern. The intrinsic resistance to antibiotics can be used for the identification of rhizobial strains that occupy nodules in studies designed to evaluate the ecological competitiveness (Kremer and Peterson, 1982). In addition, the pattern of antibiotics resistance has been used to identify diversity among strains of rhizobia. As a result, it could be used as supplementary diagnostic character for different rhizobial strains (Amarger et al., 1997).

A comparable behavior was observed with heavy metals (Table 3). More than 16 and 66% of isolates showed good tolerance to zinc and chromium, 23% to manganese at acidic pH (5.0). Other heavy metals were found to be more inhibitory since none of isolates exhibited an intrinsic resistance to them. However, the effective concentrations available to these isolates will be lower than those added since metal ions complex with agar and media components. Therefore, the tested rhizobia may be sensitive to much lower concentrations in soils. Though metals concentrations in soils origin of the isolates were not determined their metal toxicity tolerance might be related their adaptation at their soil of isolation sites. In fact, this requires further investigation. The heavy metal resistance traits of the rhizobial isolates would be used as invaluable positive markers during genetic studies (Küçük and Kıvanç, 2008). The high level of Zn and Cr resistance suggest that these metals could

be used as selective agents for some *Rhizobium strains* (Küçük and Kıvanç, 2008; Sinclair and Eaglesham, 1984)

C-Sources and N-Sources utilization

Most of the lentil rhizobia strains were able to catabolize a large variety of carbon substrates (Table 3). All tested strains grew on D-glucose and D-mannitol. None of them utilized citrate and more than 70% of the isolates utilized all tested carbohydrates as C-source. Generally, the rhizobial strains exhibited a large diversity in utilizing different carbohydrates. All tested isolates were unable to utilize citrate as sole source of carbon. Graham and Parker (1964) found that utilization of citrate as sole sources of carbon was restricted to slow-growing rhizobia. As reported by earlier studies (Graham and Parker, 1964; Sadowsky et al., 1983), fast-growing rhizobia were able to grow on a large variety of carbon substrates whereas slow-growing rhizobia were more limited in their ability to use diverse carbon sources. However, our results show the majority of tested lentil rhizobia were able to use a broad range of carbohydrates which is in agreement with the conclusion. It is very interesting to notice that the types of carbohydrates utilized also vary from monosaccharides to polysaccharides like starch and cellulose among lentil rhizobia. Similarly, other studies (Glenn and Dilworth, 1981; Sadowsky et al., 1983) observed that disaccharides such as cellobiose, lactose, maltose, trehalose and sucrose, and trisaccharide such as raffinose were catabolized by fast-growing strains of rhizobia.

Lentil rhizobia also exhibited diversity in utilizing different amino acids and vitamins as sole N-sources (Table 3).Serine and riboflavin were utilized by all isolates where as phenylalanine and glycine were not utilized by any isolates tested. Except tryptophan and niacin more than 80% of the isolates metabolized the remaining N-sources tested. This is inline with the previous studies (Amarger et al., 1997; Küçük and Kıvanç, 2008). The ability of isolates to utilize wide range of N-sources would give more survival advantage in the soil and it is one of the desirable characteristics for isolates selected for field studies.

PGP properties of the isolates

Lentil nodulating rhizoia showed very interesting characteristics such as auxin production and inorganic phosphate solubilization (Table 2). Out of all tested isolates 36.7% of them were auxin producer while only 16.7% were insoluble inorganic phosphate solubilizer with solubilization index ranging from 1.3 to 1.14 which was recorded for isolate LR25 and LR15, respectively. Several studies reported that different strains of *R. leguminosarum bv.viciae* are endowed with these characteristics (Antoun et al., 1998; Alikhani et al., 2006; Sajjad et al., 2008; Alikhani and Yakhchali, 2009; Etesami et al., 2009). Contrary with the previous study (Antoun et al., 1998) none of the tested isolates showed any antagonistic activity against test fungal pathogens in dual culture assay. Since rhizobia enjoy saprophytic life in soil when there is no legume host they non-specifically attracted to the roots of non-legume crops and nourish root exudates in the rhizosphere (Dowling and Broughton, 1986). This would give us the advantage of increasing the yield of non-legume crops by inoculating legume with symbiotically effective rhizobia which have PGP characteristics such as IAA production and PSB activity (Matiru and Dakora, 2004). Several studies demonstrated that such kind of rhizobia would increase the yield of non-legume crops grown in rotation or mixed cropping with legumes (Antoun et al., 1998; Alikhani and Yakhchali, 2009; Etesami et al., 2009). Thus, root colonization pattern and PGP activity of such isolates when inoculated to wheat, teff and barley which is grown in rotation with lentil under Ethiopian conditions needs to be investigated.

Based on the result of our studies the following isolates LR1, LR2, LR6, LR8, LR23, LR24, LR25 and LR26 are highly recommended for field trail and ecological competitiveness studies under different Ethiopian soil and climatic conditions (Table 3). These isolates have exhibited interesting features such as wide range of carbon-sources and nitrogen sources utilization, tolerance of acidic pH, metal toxicity, antibiotics, PGP features such as auxin production and inorganic phosphate solubilization, and highly effective nitrogen fixation. In general, from the present study, it can be concluded that Ethiopian soils harbor highly efficient nitrogen-fixing lentil nodulating rhizobia which are diverse in their morphological, physiological and symbiotic characteristics. Our study also indicated that some of these isolates possess special characteristics such as IAA production and phosphate solubilization activities which can make them candidate for multipurpose inoculants production for the lentil production system in Ethiopia. During this study methods used for characterizing and distinguishing rhizobial strains were morphological, physiological and symbiotic. However, these traditional methods of rhizobial characterization frequently fail to identify strains within a species. Hence, such kinds of study must be substantiated by PCR based molecular methods such as RFLP, RAPD, AFLP and 16S rRNA sequence analysis so as to obtain a better understanding of microbial diversity and strain identification.

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